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IN VIVO AND *IN VITRO* IMMUNOMODULATORY AND ANTI-INFLAMMATORY EFFECTS OF TOTAL FLAVONOIDS OF ASTRAGALUS

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Abstract

Background: *Astragali Radix* has long been used to improve immune function in traditional Chinese medicine. However, its main active components and potential immunomodulatory or anti-inflammatory activities have not been elucidated. In the present study, the immunomodulatory and anti-inflammatory activities of total flavonoids of *Astragalus* (TFA) isolated from *Astragali Radix* were evaluated by using *in vivo* animal models and *in vitro* cell models.

Materials and Methods: The *in vivo* Immunomodulatory and anti-inflammatory activities of TFA were assessed by measuring macrophage phagocytic index, delayed type hypersensitivity, serum hemolysin level and immune organ index in mice, ear edema test in mice, paw edema test in rats, vascular permeability test in mice and granuloma test in rats. The *in vitro* Immunomodulatory and anti-inflammatory activities of TFA were assessed by examining its effect on cytokine and mediator production in un-stimulated and LPS-stimulated murine RAW 264.7 macrophages.

Results: The results of *in vivo* experiments showed that TFA enhanced macrophage phagocytic index, delayed type hypersensitivity, serum hemolysin level and immune organ index in mice, and attenuated mouse ear edema, rat paw edema, mouse vascular permeability and rat granuloma formation. The results of *in vitro* experiments showed that TFA stimulated the production of NO and cytokine TNF-α, IL-1β, IL-6 and IFN-γ in un-stimulated RAW 264.7 macrophages, and inhibited the overproduction of these inflammatory mediators in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner without exerting cytotoxicity.

Conclusion: These results of this study indicate that TFA have potential immunostimulatory and anti-inflammatory effects.

Key words: Total flavonoids of *Astragalus* (TFA), Immunomodulation, Anti-inflammation, *In vivo*, *In vitro*

Introduction

Astragali Radix is the dried root of *Astragalus membranaceus* Bunge. It is one of the oldest and most frequently used crude drugs for oriental medicine in China, Korea, Japan and other Asian countries. *Astragali Radix* is a qi supplement drug in traditional Chinese medicine (TCM) and has many effects such as lifting the sunken yang, enhancing the defensive energy and superficial resistance, promoting pus discharge and tissue regeneration, and inducing diuresis to cure edema (Kim et al., 2003; Veterinary Pharmacopoeia Committee of PRC, 2011).

Modern pharmacological researches and clinical practices demonstrate that *Astragali Radix* possesses a wide spectrum of activities, including immune-modulation, cardiovascular protection, hepatoprotection, antihyperglycemic effect, anti-tumor, anti-oxidation, anti-inflammation, antiviral activity, antiallergic rhinitis and neuroprotection (McKenna et al., 2002; Cho et al., 2007; Ryu et al., 2008; Chu et al.,

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2010; You and Leung, 2011). *Astragali Radix* is a complex and multi-component system, three groups of constituents namely saponins, polysaccharides and flavonoids have been described as the principle active constituents of *Astragali Radix*. A number of bioactivity studies of saponins and polysaccharides of *Astragalus Radix* have been published (Liu et al., 2010; Qiu et al., 2010). However, there are few reports on the bioactivity of the total flavonoids of *Astragalus* (TFA). Flavonoids are found in most parts of the plants and have been attributed with multiple biological activities such as antioxidative, anticarcinogenic, anti-inflammatory, antibacterial, antiviral and immune-stimulating effects (Cazarolli et al., 2008; Landis-Piwowar and Dou, 2008; Rahimi et al., 2010; Szliszka et al., 2011). TFA are the main active component isolated from *Astragali Radix* and have been established as one of the most beneficial components of *Astragali Radix*. Series of studies have shown that TFA can offer biological system resistance to injury, and have antioxidant, antimutagenic, antitumor, inhibition of atherosclerosis and other biological effects (Zhang and Wang, 2010; Zhang et al., 2012). However, no report has been issued on the Immunomodulatory/anti-inflammatory effects and potential mechanisms of TFA.

To gain insight into the role of TFA in modulating the immune response and the potential mechanism, in this study, the *in vivo* Immunomodulatory and anti-inflammatory activities of TFA were assessed by measuring macrophage phagocytic index, delayed type hypersensitivity, serum hemolysin level and immune organ index in mice, ear edema test in mice, paw edema test in rats, vascular permeability test in mice and granuloma test in rats. The *in vitro* Immunomodulatory and anti-inflammatory activities of TFA were assessed by examining its effect on cytokine and mediator production in un-stimulated and LPS-stimulated murine RAW 264.7 macrophages.

Materials and Methods

Reagents

Carrageenan, LPS (*Escherichia coli* 055:B5), dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (DXM) was purchased from Changle Pharmaceutical Co. (Henan, China). Levamisole hydrochloride was purchased from Jinghua Pharmaceutical Co. (Sichuan, China). Cyclophosphamide (CTX) was purchased from Hengrui Pharmaceutical Co. (Jiangsu, China). India ink was purchased from Dulai Biotechnology Co. (Jiangsu, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and other reagents for cell culture were obtained from Life Technologies Inc. (Grand Island, NY, USA). Mouse TNF-α, IL-1β, IL-6 and IFN-γ ELISA kits were purchased from Biolegend Inc. (San Diego, CA, USA). All the other chemicals (analytical grade) used in this study were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China).

Extraction, Purification and Identify of TFA

The dried roots of *Astragalus membranaceus* Bunge were collected from Jilin, China, in September 2012. This plant was identified macroscopically and microscopically according to the Pharmacopoeia of China, and the voucher specimen (no. 120922) was deposited at the Herbarium of Yanbian Unviersity (Yanji, Jilin, China). TFA was provided by Shanghai Yuanye Biotechnology Co. (Shanghai, China). Briefly, the dried roots of *Astragalus membranaceus* Bunge were sliced and macerated in 95% ethanol and then extracted. The ethanol extraction was repeated 3-4 times and the pooled extract then was concentrated to a specific gravity of 1.35 by evaporation. The concentrated solution was extracted with ethyl acetate. The concentrated ethyl acetate extract was further separated by silica gel columns. The main chemical components of TFA were identified by ultraviolet spectrum, nuclear magnetic resonance, and mass spectra. It was defined that TFA contained six main chemical components including β-sitosterol, daucosterol, formononetin, calycosin, formononetin-7-O-β-D-glucopyranoside, and calycosin-7-O-β-D-glucopyranoside (Figure 1). It was consistent with previous reports (Wang et al., 2012; Xiao et al., 2005). The purity of TFA exceeded 90%, quality control standard was 90±10%.

Animals

BALB/c mice (18-22 g, SPF grade) and Wistar rats (200-220 g, SPF grade) were purchased from the Center of Experimental Animals of Yanbian Medical College of Yanbian University (Yanji, Jilin, China). The animals were kept in isolator cages and received food and water *ad*

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libitum. Before experimentation, the animals were allowed to adapt to the experimental environment for a minimum of one week. All animal experimental procedures were performed in accordance with the guidelines of the Ethical Committee for the Experimental Use of Animals at Yanbian University (Yanji, Jilin, China).

Immunomodulatory Effects of TFA *in Vivo*

Determination of Macrophage Phagocytic Index in Mice. The phagocytic activity of macrophage was performed by carbon-clearance test in mice. Mice were randomly divided into six groups (n=10): normal group, model group, TFA groups (at doses of 25, 50 and 100mg/kg, respectively), and positive treatment group. Mice from TFA groups were administered intragastrically with TFA at 25, 50 and 100 mg/kg in 0.5% Sodium Tvlose once per day for 7 days consecutively. Mice from normal group were administered intragastrically with 0.5% Sodium Tvlose. Mice from positive treatment group were given intragastrically with Levamisole hydrochloride at 30 mg/kg. On days 2, 4 and 6, mice were injected intraperitoneally with CTX at 40 mg/kg (excepting for normal group). At 1 h after the last administration, mice were injected via caudal vein with diluted India ink (10ml/kg), 20 µl of blood was collected from tail of each mouse at 2 min and 10 min after injection, and added into 2 ml of Na_2CO_3 solution (1 mg/ml), mixed and the absorbance was measured at 650 nm on a microplate reader. The liver and spleen were removed and weighed. Phagocytic index=[body weight/(liver weight+spleen weight)]×$[(lgOD_2-lgOD_{10})/(t_{10}-t_2)]^{1/3}$. OD_2=the absorbance of 2 min after injection of India ink, OD_{10}=the absorbance of 10 min after injection of India ink, t_{10}=10 min, t_2=2min.

Determination of Delayed Type Hypersensitivity (DTH) in Mice. DTH was determined by footpad swelling test. Mice grouping and experimental design were the same as above. On day 2 of drug administration, mice were sensitized via intraperitoneal injection of 0.2 ml of 2% SRBC. On day 7 of the administration, mice were injected subcutaneously with 20 µl of 20% SRBC into the right rear footpads to induce the attack. After 24 h, thicknesses of left and right rear footpads were measured again with a vernier caliper. DTH degree was represented by difference in thickness of left and right rear footpads before and after the attack.

Determination of Serum Hemolysin Level in Mice. Mice grouping and experimental design were the same as above. On day 3 of drug administration, mice were injected intraperitoneally with 0.2 ml of 2% SRBC. At 24 h after the last administration, blood was drawn from the brachial plexus, serum was obtained by centrifugation and diluted $100^{\times}$. 1 ml of mouse serum, 0.5 ml of 10% SRBC, 0.5 ml of 10% complement were mixed, then kept in water bath for 30 min at 37°C and terminated the reaction in ice bath. After centrifugated, the supernatant was collected and the absorbance was measured at 540 nm on a microplate reader.

Determination of Immune Organ Index. Mice grouping and experimental design were the same as above. On 7 day of drug administration, the mice were weighed and killed by cervical dislocation. The thymus and spleen were excised and weighed. The thymus and spleen indices were calculated as: thymus or spleen index (mg/g)=weight of thymus or spleen/body weight.

Anti-inflammatory Effects of TFA *in Vivo*

Assessment of Dimethylbenzene-induced Ear Edema in Mice. Mice were randomly divided into five groups (n=10): control group, TFA groups (at doses of 25, 50 and 100 mg/kg, respectively), and positive group. Mice from TFA groups were administered intragastrically with TFA at 25, 50 and 100 mg/kg in 0.5% Sodium Tvlose once per day for 5 days consecutively. Mice from control group were administered intragastrically with 0.5% Sodium Tvlose. Mice from positive group were given intraperitoneally with DXM at 2.5 mg/kg once 1 h prior to dimethylbenzene administration. One hour after the last drug administration, ear edema was induced by topical application of dimethylbenzene on the right ear of each mouse. The left ear was considered as control. One hour after dimethylbenzene treatment, mice were sacrificed under anesthesia and discs of 6.0 mm diameter were punched out from each ear and weighed. The ear edema was measured by subtracting the weight of the left ear from that of the right.

Assessment of Carrageenan-induced Paw Edema in Rats. Rats were randomly divided into five groups (n=10): control group, TFA groups (at

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doses of 25, 50 and 100 mg/kg, respectively), and positive group. The methods of drug administration were the same as above. One hour after the last administration, acute paw edema was induced by sub plantar injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. Paw size was measured by wrapping a piece of cotton thread round the paw of each rat and recording the length of the thread, the paw circumference, by use of a metric ruler. Paws were measured immediately prior to and 4 h after carrageenan injection.

Assessment of Acetic Acid-induced Vascular Permeability in Mice. Mice grouping and experimental design were the same as above. One hour after the last drug administration, mice were injected intravenously with 0.2 ml of 2% Evans blue. Simultaneously, 0.7% (v/v) acetic acid in saline (10 ml/kg) was injected abdominally. After 30 min, the mice were sacrificed under anesthesia, and then the abdominal cavity of each mouse was rinsed with 5 ml of normal saline solution. The recovered wash solution was centrifuged, and the absorbance of the supernatant was measured at 590 nm on a microplate reader.

Assessment of Cotton Pellet-induced Granuloma in Rats. A single needle incision was used to implant sterile cotton pellets into both axillae regions in rats under ether anesthesia. Then the rats were randomly divided into the following five groups (n=10) for the treatment once a day for 7 days: control group (0.5% Sodium Tvlose, intragastrically), TFA groups (at doses of 25, 50 and 100 mg/kg, intragastrically), and positive group (2.5 mg/kg DXM, intraperitoneally). On day 8, the cotton pellets surrounded by granuloma tissue were carefully dissected. The wet pellets were weighed and dried at 60℃ to constant weight. The granuloma formation was measured by the increase in dry weight of the pellets.

Cell Culture

The RAW 264.7 murine macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Cell Viability Assay

Cell viability was determined by MTT assay. RAW 264.7 cells (4×10^5 cells/ml) were plated onto 96-well plates and incubated overnight. The cells were treated with various concentrations of TFA (0-150 μg/ml, dissolved in 0.1% DMSO) for 24 h. 50 μl of MTT was added to each well, and the cells were incubated for another 4 h at 37°C with 5% CO_2. MTT was removed, and the cells were lysed with 100 μl/well DMSO. The optical density (OD) of each well was measured at 570 nm on a microplate reader.

Cytokine Assays

RAW 264.7 cells (4×10^5 cells/ml) were plated onto 96-well plates and treated with 10, 25 and 100 μg/ml of TFA (dissolved in 0.1% DMSO), either with or without 1 μg/ml of LPS for 24 h. Cell-free supernatants were collected and assayed for cytokines. The concentrations of TNF-α, IL-1β, IL-6 and IFN-γ in the cell supernatants were measured using ELISA kits according to the manufacturer's instructions.

Nitrite Measurement

The nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. RAW 264.7 cells (4×10^5 cells/ml) were plated onto 96-well plates and treated with 10, 25 and 100 μg/ml of TFA (dissolved in 0.1% DMSO), either with or without 1 μg/ml of LPS for 24 h. The cell supernatants were collected and assayed for NO production using Griess reagent. Briefly, the samples were mixed with equal volume of Griess reagent and incubated at room temperature for 15 min. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined using a sodium nitrite serial dilution standard curve.

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Statistical Analysis

All values are expressed as means ± standard error of the mean (SEM) of results obtained from at least three independent experiments. Differences between mean values of normally distributed data were assessed with one-way analysis of variance (ANOVA) and two-tailed Student's *t*-test. A *p*-value <0.05 was considered significant.

Results and Discussion

Immunomodulatory Effects of TFA *in Vivo*

Effects of TFA on Macrophage Phagocytic Function in Mice. Phagocytosis is the process by which certain body cells, collectively known as phagocytes, ingest and remove microorganisms, malignant cells, inorganic particles and tissue debris. Phagocytes (including neutrophils, monocytes and macrophages) are key participants in the innate immune response among the earliest cell types to respond to invasion by pathogenic organisms (Birk et al., 2001). Macrophages are ancient and phylogenetically conserved cells in all multicellular organisms and represent the first line of host defense after the epithelial barrier. So the phagocytotic function of mononuclear phagocytic system is regarded as the marker to detect non-specific immunity (Hyang et al., 2001) and macrophage phagocytic index could reflect the phagocytotic function. In this study, the effect of TFA on macrophage phagocytic index in mice was estimated by the carbon clearance method. As shown in Table 1, macrophage phagocytic index of mice was significantly decreased in model group compared with normal group ($p<0.01$). However, TFA at 25, 50 and 100 mg/kg or positive drug significantly elevated macrophage phagocytic index compared with model group ($p<0.05$ or 0.01). It suggests that TFA may enhance non-specific immunity by increasing the phagocytosis of macrophages and result in the initiation of mononuclear phagocytic system function of immune reaction against foreign materials.

Effects of TFA on Delayed Type Hypersensitivity in Mice. Delayed type hypersensitivity reaction is a protective localized cell-mediated immune-inflammatory response, primarily against intracellular pathogens. Upon antigen presentation, T-lymphocyte may become sensitized lymphocyte and will generate a regional abnormal reactive inflammation. This allergic inflammation is delayed and characterized by cell degeneration and necrosis (Heriazon et al., 2009). In this study, SRBC-induced delayed type hypersensitivity was used to assess the effect of TFA on cellular immunity. The effect of TFA on delayed type hypersensitivity was determined by the footpad swelling method. As shown in Table 1, significant decrease of footpad swelling was observed in model group compared with normal group ($p<0.01$), which indicated the feasibility of the present immunosuppressed model. However, TFA at 25, 50 and 100 mg/kg or positive drug significantly increased footpad swelling rate compared with the model group ($p<0.05$ or 0.01), which indicated that TFA might strengthen cell-mediated immune function by increasing DTH footpad swelling.

Effects of TFA on Serum Hemolysin Level in Mice. Serum hemolysin antibody is a reliable indicator reflected by the level of humoral immunity, and secreted by B-lymphocytes when stimulated by a variety of antigens (Tsai et al., 2005). So we evaluated the level of serum hemolysin in response to SRBC in mice. As shown in Table 1, a significant decrease of serum hemolysin level was observed in model group compared with normal group ($p<0.01$). However, TFA at 50 and 100 mg/kg or positive drug significantly enhanced serum hemolysin level compared with model group ($p<0.05$). The results indicated that TFA might strengthen humoral immune function by enhancing serum hemolysin level.

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Table 1: Effects of TFA on macrophage phagocytic index, delayed type hypersensitivity, serum hemolysin level and immune organ index in mice

Group	Phagocytic index	Increase in thickness of foodpad (mm)	Serum hemolysin level (OD value)	Thymus index (mg/g)	Spleen index (mg/g)
Normal	3.85±0.40	1.45±0.14	0.28±0.11	2.79±0.33	3.46±0.42
Model	2.83±0.37$^{\#\#}$	0.85±0.05$^{\#\#}$	0.15±0.03$^{\#\#}$	1.32±0.17$^{\#\#}$	2.05±0.21$^{\#\#}$
25 mg/kg of TFA	3.14±0.35*	1.03±0.03*	0.21±0.09	1.63±0.15*	2.44±0.15
50 mg/kg of TFA	3.30±0.28*	1.13±0.08**	0.25±0.04*	2.14±0.18*	2.81±0.22**
100 mg/kg of TFA	3.43±0.29**	1.08±0.03**	0.23±0.08*	2.15±0.21*	2.74±0.18*
Positive	3.42±0.37**	1.10±0.04**	0.24±0.08*	2.27±0.18**	2.94±0.24**

Note: Data are presented as means ± SEM (n=10). $^{\#\#}p<0.01$ vs. control group; $^{*}p<0.05$, $^{**}p<0.01$ vs. model group.

Table 2: Effects of TFA on ear edema in mice, paw edema in rats, vascular permeability in mice and granuloma in rats

Group	Ear edema (mg)	Paw edema (mm)	OD value	Weight of granuloma (mg)
Control	14.32±2.04	6.09±0.92	0.42±0.23	30.12±5.42
25 mg/kg of TFA	10.25±2.12	4.50±0.46*	0.35±0.18	22.35±3.78*
50 mg/kg of TFA	6.84±1.78**	3.61±0.51**	0.30±0.22*	14.75±2.86**
100 mg/kg of TFA	5.03±2.30**	2.82±0.27**	0.24±0.16**	12.55±2.41**
Positive	4.70±1.81**	3.07±0.38**	0.21±0.13**	12.07±2.65**

Note: Data are presented as means ± SEM (n=10). $^{*}p<0.05$, $^{**}p<0.01$ vs. control group.

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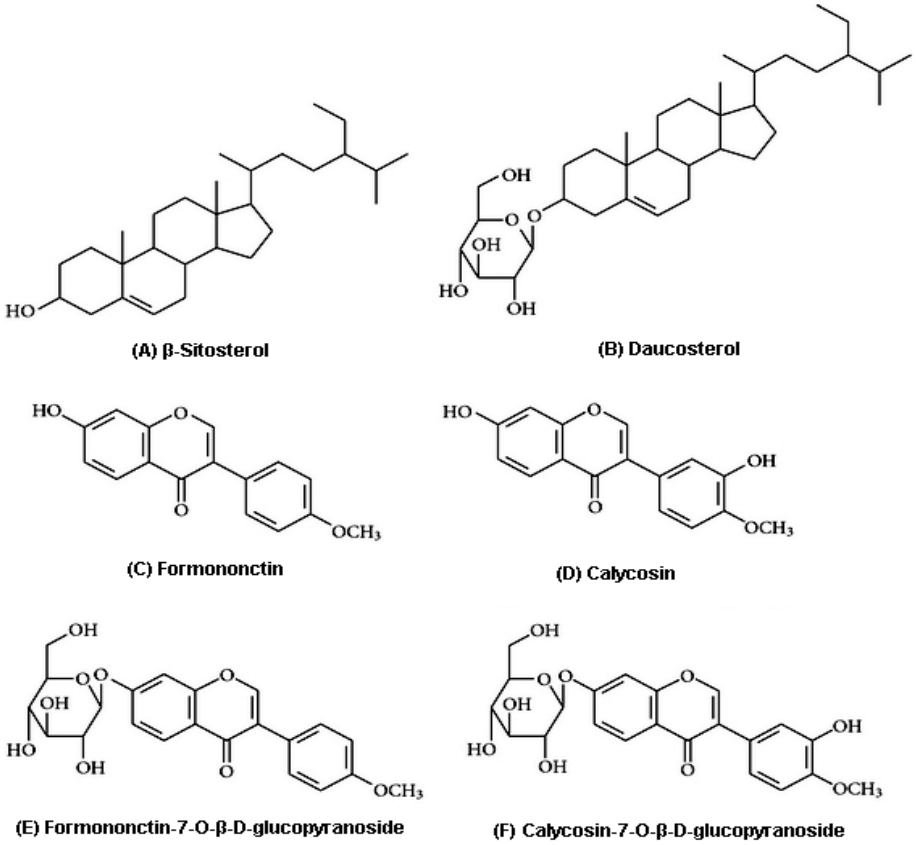

Figure 1: The six main chemical components of TFA

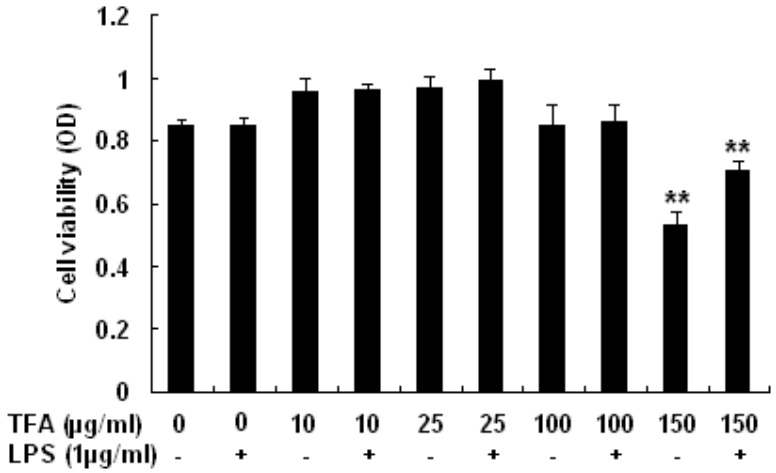

Figure 2: Effect of TFA on the viability of RAW 264.7 cells. RAW 264.7 cells were incubated with TFA (0-150 μg/ml) in the absence or presence of LPS (1 μg/ml) for 24 h. Cell viability was determined by MTT assay. Data are presented as means ± SEM of three independent experiments. ***p*<0.01 vs. control group.

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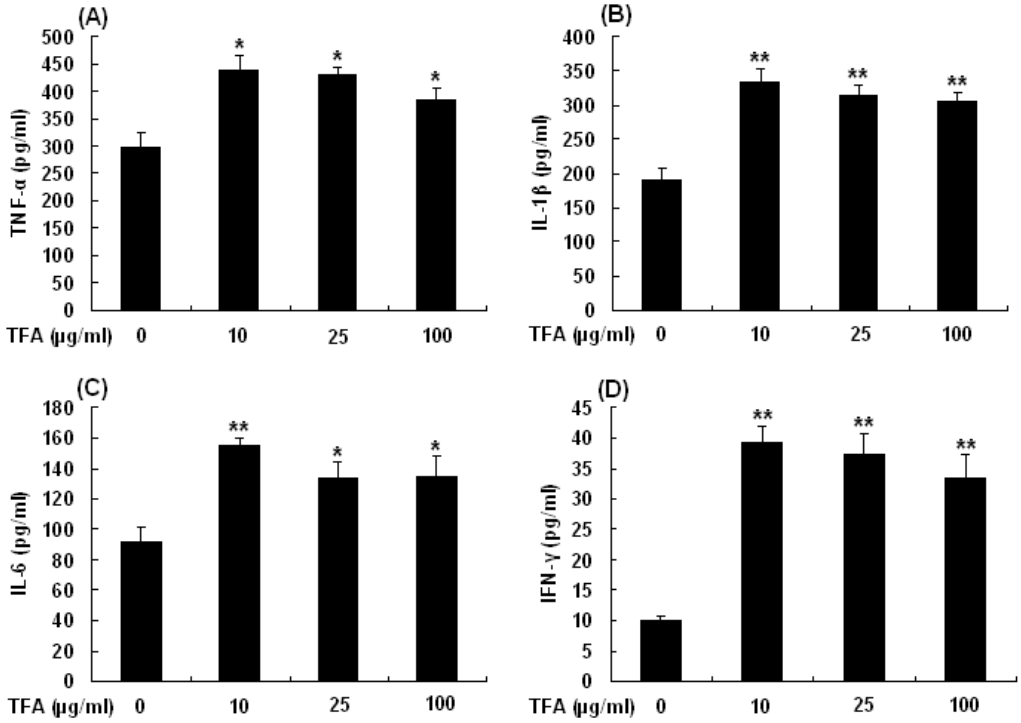

Figure 3: Effect of TFA on production of TNF-α (A), IL-1β (B), IL-6 (C) and IFN-γ (D) in un-stimulated RAW 264.7 cells. The cells were treated with different concentrations (10, 25, 100 μg/ml) of TFA for 24 h. Control group was treated in the absence TFA. The values are means ± SEM of three independent experiments. *p<0.05, **p<0.01 vs. control group.

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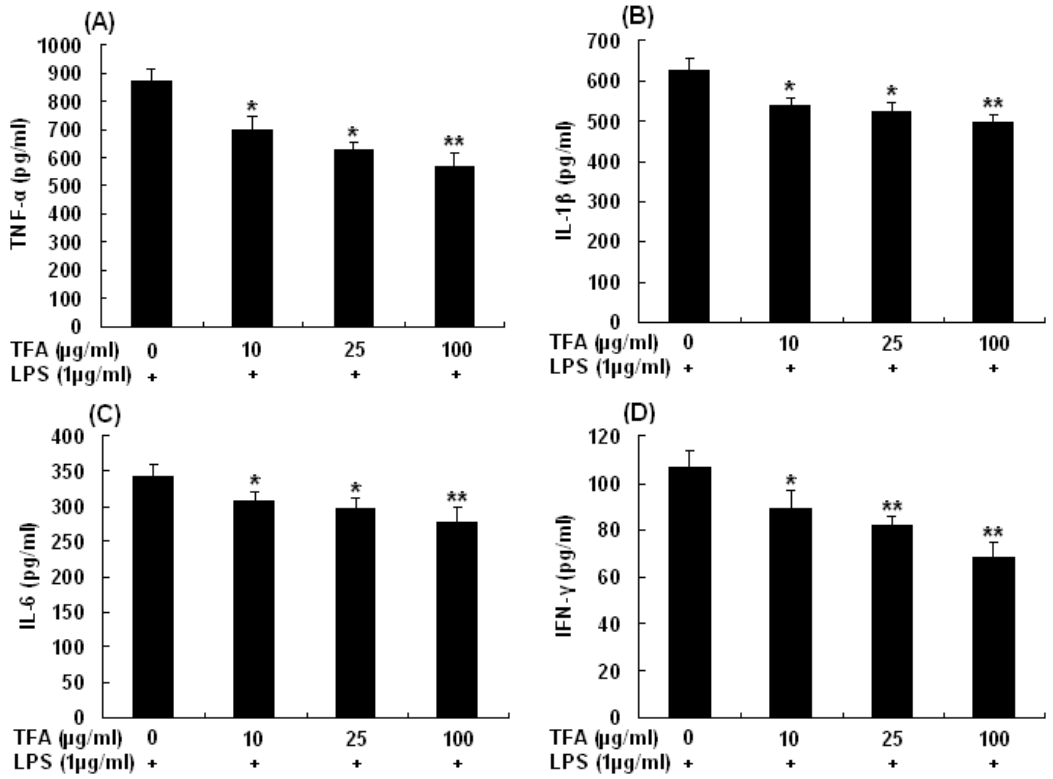

Figure 4: Effect of TFA on production of TNF-α (A), IL-1β (B), IL-6 (C) and IFN-γ (D) in LPS-stimulated RAW 264.7 cells. The cells were treated with different concentrations (10, 25, 100 μg/ml) of TFA and 1 μg/ml of LPS for 24 h. LPS group was treated with LPS only. The values are means ± SEM of three independent experiments. *p<0.05, **p<0.01 vs. LPS group.

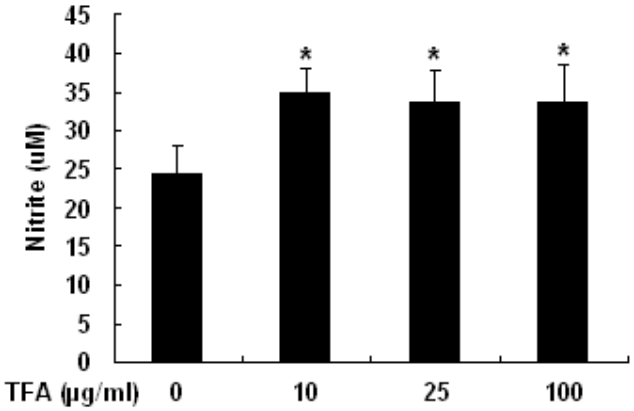

Figure 5: Effect of TFA on production of NO in un-stimulated RAW 264.7 cells. The cells were treated with different concentrations (10, 25, 100 μg/ml) of TFA for 24 h. Control group was treated in the absence TFA. The values are means ± SEM of three independent experiments. *p<0.05 vs. control group.

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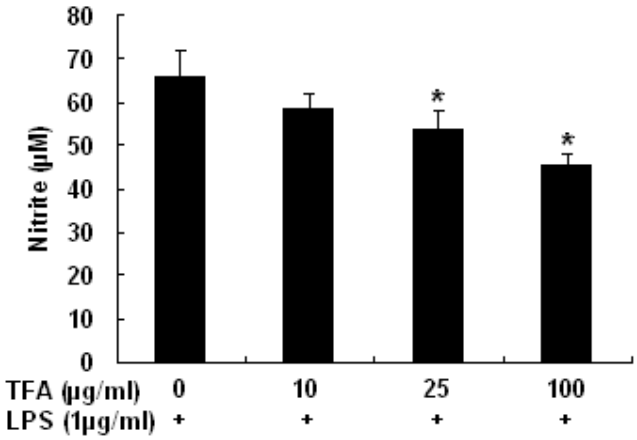

Figure 6: Effect of TFA on production of NO in LPS-stimulated RAW 264.7 cells. The cells were treated with different concentrations (10, 25, 100 μg/ml) of TFA and 1 μg/ml of LPS for 24 h. LPS group was treated with LPS only. The values are means ± SEM of three independent experiments. *p<0.05 vs. LPS group.

Effects of TFA on Immune Organ Index in Mice. Thymus and spleen are important immune organs and can relatively reflect the immune function of animals. The immunomodulatory effect is closely related to the change of immune organ index. Thus, spleen index and thymus index are considered as the most elementary and conventional index, which have been generally used to evaluate the whole immune state of the organism (Gao et al., 2013). In this study, the effects of TFA on spleen index and thymus index were investigated. As shown in Table 1, thymus and spleen indices of mice treated with CTX significantly decreased compared with normal group (p<0.01), which displayed that the immunosuppressed model was successfully built. However, TFA or positive drug significantly increased the thymus and spleen indices of mice compared with model group (p<0.05 or 0.01).

Anti-inflammatory Effects of TFA *in Vivo*

Effect of TFA on Dimethylbenzene-induced Ear Edema in Mice. Edema is a typical symptom of inflammation not only in systemic inflammation, but also in local inflammation (Liu et al., 2010). The dimethylbenzene-induced ear edema in mice is a preliminary and widely used model for screening potential anti-inflammatory drugs (He et al., 2013). Topical application of dimethylbenzene significantly elicited an inflammatory response in mice, in this process of inflammation, vasodilatation brings about plasma extravasations and inflammatory mediator releases, which trigger the acute inflammation response (Eddouks et al., 2012), as judged by edema formation determined by the increase in the weight of the ear. As shown in Table 2, topical application of dimethylbenzene markedly elicited an inflammatory response in mice as judged by ear edema formation (p<0.01). However, compared with the dimethylbenzene-induced control group, TFA at 50 and 100 mg/kg significantly inhibited ear edema (p<0.05 or 0.01). DXM also significantly inhibited dimethylbenzene-induced ear edema in mice (p<0.01). The inhibition of ear edema indicated that TFA attenuated vasodilatations and plasma extravasations of neurogenic inflammation, which are crucial in controlling the early stage of acute inflammation.

Effect of TFA on Carrageenan-induced Paw Edema in Rats. The carrageen-induced paw edema is acute, well researched and highly reproducible model, and has been commonly employed to assess the anti-edematous effect of natural products (Kang et al., 2010). To estimate the effect of TFA on acute inflammation, we also used carrageenan-induced rat paw edema as an assay to reflect edema that occurs during the early stages of acute inflammation (Matsuda and Tanihata, 1992). As shown in Table 2, the rat paw edema obviously increased after carrageenan

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induction. Compared with the carrageenan-induced control group, TFA at all doses suppressed paw edema after 4 h of carrageenan induction in a dose-dependent manner (p<0.05 or 0.01). DXM also significantly inhibited paw edema induced by carrageenan compared with the control group (p<0.01). It is consistent with the result of dimethylbenzene-induced ear edema in mice.

Effect of TFA on Acetic Acid-induced Vascular Permeability in Mice. Increased vascular permeability is an early and important vascular event in the inflammatory response (Khor et al., 2009). Acetic acid challenge brings about increases in the level of mediators such as prostaglandin, serotonin, and histamine in peritoneal fluids, which in turn lead to a dilation of the capillary vessels and an increase in vascular permeability (Eddouks et al., 2012). Thus, the vascular permeability test was carried out to further demonstrate the anti-inflammatory effects of TFA, which exhibited significant inhibitory effects on the increased vascular permeability induced by acetic acid in mice. The vascular permeability was represented by the amount of Evans blue extruded into abdominal cavity, which was measured by the OD value of the supernatant. As shown in Table 2, compared with the acetic acid-induced control group, TFA at 50 and 100 mg/kg exhibited significant inhibitory effect on the increased vascular permeability induced by acetic acid in mice in a dose-dependent manner (p<0.05 or 0.01). DXM also significantly inhibited acetic acid-induced vascular permeability in mice (p<0.01). This result consolidates that the anti-inflammatory effect of TFA in the acute phase of inflammation associates with prevention of vasodilation and may be mediated by inhibiting the releases of inflammatory mediators.

Effect of TFA on Cotton Pellet-induced Granuloma in Rats. Granulomatous inflammation is a focal chronic inflammatory response to tissue injury evoked by a poorly soluble substance characterized by the accumulation and proliferation of leukocytes, principally of the mononuclear type (Hirsh and Johnson, 1984). The induction of granuloma formation by inserting cotton pellet subcutaneously into a rat is widely used to assess the effect of anti-inflammatory drugs on the transudative and proliferative phases of chronic inflammation (Eddouks et al., 2012; Swingle and Shideman, 1972). The weight of granulation is an index of granuloma formation, which is used to evaluate the chronic inflammation. As shown in Table 2, cotton pellet induced granuloma formation in the cotton pellet-induced control group, TFA at 25, 50 and 100 mg/kg inhibited granuloma formation surrounding cotton pellets compared with the cotton pellet-induced control group in a dose-dependent manner (p<0.05 or 0.01). DXM also significantly inhibited cotton pellet-induced granuloma in rats (p<0.01).

Effect of TFA on Cell Viability

The potential cytotoxicity of TFA on RAW 264.7 macrophages was evaluated by the MTT assay after incubating cells for 24 h in the absence or presence of LPS (Figure 2). The result showed that TFA at concentrations from 0 to 100 μg/ml had no cytotoxic effect on RAW 264.7 cells. However, the viability of the cells was significantly reduced when 150 μg/ml of TFA was used (p<0.01). With this result, the concentrations of TFA applied never exceeded 100 μg/ml for subsequent experimentation.

Effects of TFA on Cytokine Production in Un-stimulated and LPS-stimulated RAW 264.7 Macrophages

RAW 264.7 murine macrophage cell line has been used to characterize the immunomodulatory action of various components *in vitro*. It releases a variety of immunomodulatory factors, including cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukocyte adhesion and NO, which leads to secondary immune response such as proliferation of T and B cells, activation of macrophages for phagocytosis, and killing of microorganisms. Among these mediators, it is widely acknowledged that cytokines such as TNF-α, IL-1β, IL-6, IFN-γ and inflammatory mediator NO can be generated in response to immunological reaction and play an important role in the regulation of immune response (Lee et al., 2003). TNF-α exhibits tumor necrosis activity and has been recognized as an important host regulatory molecule (Vilcek and Lee, 1991; Lejeune et al., 2006). TNF-α and IL-1β, considered “early response cytokines” that are produced rapidly by macrophages in response to inflammatory stimuli, upregulate expression of adhesion molecules on endothelial cells thus facilitating phagocyte margination and emigration to sites of tissue injury (Laskin and Laskin, 2001). IL-6 plays an essential role in the host immune response, acute protein synthesis and the maintenance of homeostasis (Liu et al., 2007). Therefore, variations in levels of mediators can be thought of as a marker of

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immunomodulation, and has been used for assessing the immunomodulatory activity of tested sample (Hu et al., 2008). In this study, we examined the effect of TFA on the production of cytokines in RAW 264.7 macrophages. TFA at a concentration of 10, 25 and 100 µg/ml significantly induced the secretion of TNF-α (Figure 3A), IL-1β (Figure 3B), IL-6 (Figure 3C) and IFN-γ (Figure 3D) compared with that in control group (p<0.05 or 0.01). The results showed that a dose range at which TFA was not cytotoxic and induced the secretion of cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) in normal/un-stimulated macrophages, positively modulated RAW 264.7 macrophage function, and exhibited the immunomodulatory activity in the innate immunity.

The release of inflammatory cytokines is essential for host survival from infection, and is also required for the repair of tissue injury. However, large amounts of macrophage-derived cytokines can also cause collateral damage to normal cells and are potentially lethal when the release is sufficient to cause systemic exposure (Glauser et al., 1996). They are also considered to be important initiators of the inflammatory response and mediators of the development of various inflammatory diseases (Glauser et al., 1996; Männel et al., 2000). To determine the *in vitro* anti-inflammatory effect of TFA, the levels of inflammatory cytokines TNF-α, IL-1β, IL-6 and IFN-γ in the supernatants of LPS-stimulated RAW264.7 cells were determined. Stimulation of RAW 264.7 cells with LPS resulted in significant increases in cytokine production as compared to that in control group (p<0.01, Figure 4), and TFA at 10, 25 and 100 µg/ml significantly inhibited cytokine TNF-α (Figure 4A), IL-1β (Figure 4B), IL-6 (Figure 4C) and IFN-γ (Figure 4D) production as compared to that of LPS-stimulated RAW 264.7 cells in a dose-dependent manner (p<0.05 or 0.01). The results showed that a dose range at which TFA was not cytotoxic and markedly inhibited the secretion of cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) in over-inflammatory/LPS-stimulated macrophages, negatively modulated RAW 264.7 macrophage function, and exhibited the anti-inflammatory activity of TFA. Therefore, these results demonstrate that TFA may partially exert its immunomodulatory and anti-inflammatory activities through the dual-directional modulation on the secretion of cytokines in RAW 264.7 macrophages.

Effects of TFA on NO Production in Un-stimulated and LPS-stimulated RAW 264.7 Macrophages

NO, highly unstable gas, plays an important role in diverse physiological processes, including immune responses, inflammatory and neurotransmission (Mayer and Hemmens, 1997). NO has a dual biological role. Low level or adequate concentration of NO plays a role as neurotransmitter, affects the function of immune cells, as well as tumor cells and resident cells of different tissues and organs (Palmer et al., 1998). However, high level or uncontrolled release of NO induces host cell death and inflammatory tissue damage (Zhang et al., 2009). Therefore, the production of regulating NO will be useful for the amelioration of immune and inflammatory diseases. In the present study, we found a dose range at which TFA was not cytotoxic and induced the production of mediator NO in un-stimulated RAW 264.7 macrophages, NO production was significantly induced at a concentration of 10, 25 and 100 µg/ml of TFA compared with that in control group (p<0.05) (Figure 5). However, TFA at 25 and 100 µg/ml significantly inhibited the overproduction of NO as compared to the LPS group in a dose-dependent manner (p<0.05) (Figure 6). It demonstrates that TFA may partially exert its immunomodulatory and anti-inflammatory activities through the dual-directional modulation on the secretion of inflammatory mediator NO in RAW 264.7 macrophages.

Conclusions

In conclusion, the present study showed that TFA isolated from *Astragali Radix* has significant immunostimulatory and anti-inflammatory effects *in vivo* and *in vitro*, which depends on its ability to enhance immune organ index, macrophage phagocytic index, delayed type hypersensitivity, serum hemolysin level in mice, attenuate ear edema in mice, paw edema in rats, vascular permeability in mice and granuloma formation in rats, and regulate the production of TNF-α, IL-1β, IL-6, IFN-γ and NO in RAW 264.7 macrophages. It suggests collaboration between immunostimulatory and an-inflammatory effects of TFA may contribute to the best possible immune response, which can be the integrated action mode for various medicinal efficacies of TFA. This study provides scientific support for the use of *Astragali Radix* as an oriental prescription for the amelioration of immune and inflammatory diseases. Future studies on the molecular mechanisms through which TFA exerts these effects are in progress.

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